UNIVERSITY OF SPLIT

SCHOOL OF MEDICINE

Laura Langert

PLASMA PROTEIN CARBONYLATION AS BIOMARKER FOR ESTIMATE OF AGING

Diploma thesis

Academic year:

2017/2018

Mentor:

Assoc. Prof. Ozren Polašek, MD, MPH, PhD

Split, July 2018.

UNIVERSITY OF SPLIT
SCHOOL OF MEDICINE

Laura Langert

PLASMA PROTEIN CARBONYLATION AS BIOMARKER FOR ESTIMATE OF AGING

Diploma thesis

Academic year:
2017/2018

Mentor:
Assoc. Prof. Ozren Polašek, MD, MPH, PhD

Split, July 2018.

TABLE OF CONTENTS:

ACKNOWLEDGEMENT

First of all, I would like to express my gratefulness to Ozren Polašek, who willingly excepted to mentoring my thesis. Without his support, guidance and help during the past year, the creation of my thesis would not have been possible.

Secondly, I would like to thank my boyfriend Tim, who has not only been my study partner and best friend, he was also always there for me whenever I was afraid of failing and supported me.

Last, and most importantly my biggest thanks goes to my family and especially my parents, Cordula and Martin, who made my dream of studying medicine become true. Thank you for your endless support, love and understanding. Without you, I would not be at the point in life I am right now!

This Thesis was prepared as the part of the HRZZ project CarboNyx.

1. INTRODUCTION

An increasing amount of scientific evidence proposes that oxidative stress and the resulting cellular damage are responsible for the process of aging and the evolution of various diseases (1). During the last years, a great amount of papers has been published, which document the positive correlation between an increase of certain markers of oxidative stress and the process of aging. Consequently, cells of older organisms have a higher load of oxidatively damaged macromolecules, like nucleic acids, lipids and proteins.

In the following paper, the focus will be on a specific kind of oxidative modification of proteins, namely carbonylation, and a possibility to use it as a biomarker of aging (2).

1.1. Aging

1.1.1. Definition

Aging is a highly complex biological phenomenon, affecting all biological systems, characterized by a continuous and irreversible reduced capacity of the living organism to withstand the burden of everyday life, increasing the risk of morbidity and mortality (3,4).

1.1.2. Chronological and biological age

It is common knowledge that the life expectancy (the average life span of a general population) of humans is constantly rising globally, although the underlying process of aging hasn't changed. It is attributable to several factors, including better hygiene, wealth, education and most importantly due to better public health. With increased longevity of humans, also grows the population of older people, and with this age-related sequela like diseases and impairments. That consequently leads to an increased demand of prevention and intervention strategies to support healthy aging (5,6).

It has been mentioned many times by geriatric specialists that between humans of the same chronological age, differences exist in the pace of aging. That means that the chronological age,

which is the age of a person measured from birth to a given date, is different to the biological age, which in turn measures more specifically the functional and physiological status (7-9).

The variation in aging processes between individuals is partially attributable to tissues aging at different velocities and thus leading to the fact that for any individual's chronological age the values for biological age can differ broadly. Conclusively the biological age is thought to correlate with the personal variations in longevity and timing and degree of the aging process (8, 9). "Biologically older" refers to humans whose function is poorer in comparison to others of the same chronological age, the contrary describes humans with better function and they are therefore called "biologically younger" (10).

The biological age can not only give an approximation of the individual's present general status and remaining life expectancy, it may also serve as a measure of relative fitness, help in determining the personal risk for age-related disorders and disability and to predict mortality independent from chronological age (9).

1.2. Biomarker

Over the last decades, plenty of research has been conducted on how to measure and evaluate biological age in humans.

In order to assess the rate of aging, the classical quantitative evaluation is based on the analysis of mortality curves, which means that probands have to be followed up until their death to be able to determine their biological age at any time during life. For that reason, a reliable measurement of the state of aging, namely the functional decline and a prediction of the remaining personal life expectancy at any level of a living proband is not attainable with this method. That dilemma raises the need for a workable test strategy and parameter, which would be able to determine age-related changes in body function and composition, predict the likelihood of onset morbidity and the remaining life span. That parameter is named "biological marker of aging" (4).

Various biological parameters, the so called biomarkers of aging, have been found to correlate with the physical and functional status of an organism and therefore more precisely with the biological age. Baker and Sprott defined biomarkers of aging in 1988 as follows: ''A Biomarker

of Aging (BoA) is a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict functional capability at some late age, than will chronological age'' (3).

1.3. Carbonylation

Carbonylation is a form of protein oxidation, which is modified by reactive oxygen species (11). It is a post-translational modification, produced by the addition of carbonyl groups to proteins. This process is caused by reactions between protein amino acid residues and reactive oxygen species (ROS) (12). ROS- induced post-translational protein modifications can be either of reversible or irreversible nature. Besides carbonylation, being an irreversible modification, reversible protein modifications like S-glutathionylation, S-nitrosation and methionine sulfoxidation exist. These harmless events comprise a two-fold role of modulation of protein function (redox regulation) and protection from irreversible oxidation (13-15).

Carbonylation of proteins is an irreversible and non-enzymatic transformation, which often results in a loss of function of proteins and longstanding deleterious cellular outcomes (12).

During the process of carbonylation, carbonyl groups are attached to proteins by a number of distinct oxidative pathways. Either by a direct oxidation reaction of ROS with the protein amino acid residues like proline, arginine, lysine and threonine and from the oxidative modification of the protein's backbone or by an indirect reaction. The indirect reaction refers for example to lipid peroxidation, in which lipids react with ROS or glycation/glycoxidation reaction, which apply to sugars and ROS producing reactive carbonyl derivatives, like ketones and aldehydes. These reactive carbonyl derivatives may interact with amino acids, such as cysteine, histidine and lysine in a secondary reaction, forming advanced glycation end-products (AGEs) and advanced lipoxidation end-products (ALEs) (12,16).

By reflecting cellular damage induced by multiple forms of ROS, protein carbonylation is the most widely popular biomarker for oxidative damage to proteins (17). Their products are chemically stable and uncomplicated to identify after being modified with dinitrophenylhydrazine

by different methods of immunostaining, like ELISA, fluorescence microscopy and immunoblot (18).

1.4. Reactive oxygen species and free radicals

Reactive oxygen species is the term for a number of free radicals and reactive molecules originating from molecular oxygen (19). Free radicals are molecular species, which carry an unpaired electron in an atomic orbital, thereby frequently resulting in an unstable and highly reactive state. They are able to either accept an electron from or donate an electron to a different molecule, consequently acting like reductants or oxidants (20).

In the human body, ROS are derived from endogenous or exogenous pathways, such as exposition to irradiation (x-rays), atmospheric pollutants, like ozone and cigarette smoke and industrial chemicals or xenobiotics to name only a few (21,22).

Endogenously, ROS can be generated enzymatically or non-enzymatically. The generation of ROS is primarily connected to the non-enzymatic pathway of one-electron carriers in the mitochondrial respiratory chain, in which leaking electrons are directly transferred to oxygen. That process is supposed to be the primary source of O2−• in most tissues.

In the living organism in aerobic conditions, reactive oxygen species are formed by a stepwise reduction (of electrons) of molecular oxygen (O_2) through the addition of one electron each to superoxide (O2−•), then to hydrogen peroxide (H_2O_2), afterwards producing a hydroxyl radical (•OH) and eventually formation of water (H_2O) (Figure 1).

Examples for free radicals are superoxide anion and hydroxyl radical, indicated by the dot in their chemical nomenclature. Hydrogen peroxide (H2O2) and singlet oxygen (1O2) are examples for nonradical oxidants (23).

Enzymatic sources comprise for instance NADPH oxidases on cell membranes on macrophages and polymorphonuclear cells and cytochrome P450-dependent oxygenases (19,22,24).

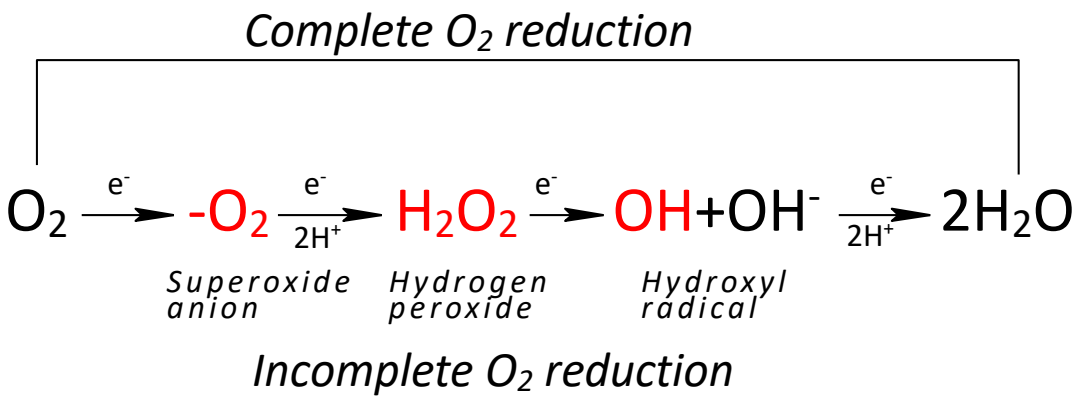

Figure 1: „Complete and incomplete reduction of molecular oxygen. The production of specific reactive oxygen species by single electron additions (e-)“ (25).

1.5. Oxidative stress and antioxidants

In 1985, Helmuth Sies was the first one to define oxidative stress, which is the imbalance between oxidants (ROS, free radicals) and antioxidants in favor of the oxidants. It can apply to situations in which the steady-state ROS concentration is either transiently or chronically increased, thereby impeding the cellular metabolism, its regulations, and inducing various post-translational modifications, such as carbonylation, which in turn may lead to consequences for cell physiology, like loss of function of the targeted cells (22,26).

Oxidative stress can be the consequence of the reduction of low molecular mass antioxidant reserves, an elevated level of endo- and exogenous ROS production and a reduced generation or an inactivation of antioxidant enzymes (26).

Oxidative stress can be divided into chronic oxidative stress and acute oxidative stress, depending on the time of the elevated ROS levels. Acute oxidative stress describes a situation in which temporarily enhanced ROS levels cannot be controlled by the antioxidant system, but after an initial increase, they return back to a stage manageable by the defense system of cells. On the contrary, in chronic oxidative stress, antioxidant mechanisms, like enhanced expression of antioxidant and related enzymes cannot balance elevated ROS amounts and the raised ROS levels will settle at a new steady level. This eventually leads to adjustments of various cellular

components and considerably perturbing homeostasis. During the last years, plenty of research has shown that chronic oxidative stress is connected to many pathologies, like cardiovascular and neurodegenerative diseases, cancer and diabetes mellitus (26).

According to Halliwell & Gutteridge in 1989, the term antioxidant defines „any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate.“ (27).

These substances can be of enzymatic and non-enzymatic nature. Antioxidant enzymes, acting like radical scavengers, establish the main antioxidant defense system of the organism (22). They achieve their action by either preventing reactive oxygen species to be formed or by eliminating them before they can exert their negative effects on vital compounds of the cell, namely DNA, lipids and proteins (28). As stated above, the basic function of the antioxidant system is to keep the ROS at an ideal level, because ROS are also believed to participate in cellular functions like redox signaling (29).

2. OBJECTIVES

The main aim of this study was to investigate the role of carbonylation in aging and its role as biomarker for estimation of age. The Thesis is a part of the Module 1 of the CarboNyx project by the Croatian Science Foundation. The module 1 was built with these goals:

Primary module objective: Measure protein carbonylation in pre-selected healthy subjects

Expected results: Determination of the pattern of changes in protein carbonylation associated with healthy ageing

Applicability: The use of protein carbonylation biomarkers as a tool to define biological age (as opposed to the chronological age), but also to use it as a mean of molecular well-being measurement (where small changes of specific protein carbonylation could reflect selective biological processes associated with stress, strain and possibly even early stages of some diseases).

3. MATERIALS AND METHODS

This study was based on a subsample of the 10,001 Dalmatians project, which is the largest research-oriented biobank in Croatia. The main objective of this program is to create a comprehensive resource for the study of genetic, environmental and social determinants of health and diseases with emphasis on chronic diseases, which are the leading cause of death in Croatia and other developed countries (30).

For the purpose of this study, we selected a subset of 50 healthy subjects from the island of Korčula. The status of a healthy subject was based on the detailed analysis of their medical records, in which there were no indications of the existence of any kind of chronical disease that could have affected the result. Their corresponding biological sample of plasma was taken from the 10.001 Dalmatians freezer and used in the analysis – no additional contact was made with the subjects, therefore no additional ethical approval was sought.

In addition to this, we had also used a number of measurements from the same dataset, allowing us the comparative analysis of the carbonylation and other measured variables. These included basic demographic data, anthropometric measurements, cognitive testing results and biochemical analysis of their serum and plasma.

3.1. Protocol

Total carbonylation and expression of the proteins in plasma samples (1D electrophoresis)

Plasma samples were depleted of most abundant proteins (albumin and IgG) using Multiple Affinity Removal Column HAS/IgG Column, 4.6 x 50 mm (Agilent Technologies). Before injection onto a MARS column, the human plasma was diluted 4X with Buffer A (Agilent Technologies). The samples were transferred to a 0.22 μm cellulose acetate spin filter (Spin-X centrifuge tube filter, Costar) and centrifuged for a 1 min at 16000 x g to remove particulates. Filtrate was collected into HPLC vial.

HPLC lines were purged with 50% isopropanol in water to ensure the removal of all unwanted salts and organics. Buffer A and buffer B (Agilent Technologies) were set as the only mobile phases, and Buffer A was used as a needle wash. Two blanks (buffer A) were injected (V_{inj} = 50 μL) and run without column by method described in Table 1.

Column was attached and equilibrated in buffer A for 4 min at a flow rate of 1 mL at room temperature. Samples were injected (V_{inj} = 50 µL) and run by method described in Table 1.

Flow-through fraction from 1.1 – 9 min (λ = 280 nm) was collected and stored at -20°C until processed further.

Table 1. LC method for 4.6 x 50 mm column.

Time (min)	**%B**	**Flow rate (mL/min)**
0.00	**0**	**0.25**
10.00	**0**	**0.25**
10.01	**0**	**1.00**
12.00	**0**	**1.00**
12.01	**100**	**1.00**
15.00	**100**	**1.00**
15.01	**0**	**1.00**
21.00	**0**	**1.00**

Buffer exchange to 1X PBS was performed using 3kDa Amicon Ultra- 4mL centrifugal filter devices (Merck). Devices were spun at 13310 x g for 40 min, filtrate was removed, 3.0 mL of 1X PBS was added to concentrated sample and were spun again at 13310 x g for 40 min. Concentrated sample was collected.

Sample concentration was measured using Bradford method and the appropriate amount of sample was diluted with 1X PBS for further staining with CF647DI-aminooxy dye (Biotium). Staining of the samples was performed over night at 4°C and 600 rpm to ensure marking of the all aldehydes and ketones.

For the SDS-PAGE were 8% resolving and 5% stacking gels prepared according to Table 2.

Table 2. Resolving and stacking gels for SDS-PAGE (4 gels in excess).

Solution components	**Resolving gel (8 %)**	**Stacking gel (5 %)**
	Component volumes (mL)	
H_2O	**11.5**	**2.8**
40 % Acrylamide mix	**6.7**	**0.83**
0.5 M Tris, pH 6.8	**/**	**1.25**
1.5 M Tris, pH 8.8	**6.3**	**/**
10 % SDS	**0.25**	**0.05**
10 % APS	**0.25**	**0.05**
TEMED	**0.015**	**0.005**

Gels were run at 70 V for half an hour until samples run through stacking gel, after which the current was increased to 130 V. The last size band of proteins on the gels was around 20-25 kDa. Gels were then stained with Serva purple dye (Serva) to determine total protein expression. Gels were scanned by Typhoon scanner (GE Healthcare) at appropriate wavelengths and data was analyzed in TotalQuant software (TotalLAb).

3.2. Statistics

All data were analyzed using descriptive and analytic statistical methods. These methods included the calculation of means and standard deviations, followed by the correlation analysis. This was based on both Spearman's rant test and Pearson test, based on the data distribution and underlying assumptions. Data fitting and trend analysis was based on the MS Excel built-in functions, which provided the opportunity to estimate the variance explained by the model. The final analytic step was based on the cross-validation approach and imputation analysis. The dataset was firstly split in two equal sized subsets, and then the chronological age information was retained for only the remaining half of the subjects. This allowed for the opportunity to impute the remaining age data, based on the gender and carbonylation levels. The same process was performed five times for each of the two sub-sets, creating a total of five complete sets of the predicted age data. This data was then used in the distribution analysis, in an attempt to provide an estimate of the possibility to use carbonylation as an ageing predictor in larger datasets. The correlation and imputations were performed in IBM SPSS (ver 21, IBM, Armonk, NY: USA).

4. RESULTS

A total of 50 randomly selected healthy subjects were included in this study. There were 18 men (36.0%) and 32 women (64%), reflecting higher percentage of women in the original study. The average age was 48.33±18.48 years for men and 47.97±14.09 years for women, what was not a significant difference (P=0.938). The overall one-dimensional carbonylation was 0.263±0.096 for men and 0.245±0.103 for women, yielding no significant difference (P=0.491).

The initial comparison of the chronological age with the overall 1D carbonylation suggested the existence of the marginally significant correlation, under a non-linear assumption (Spearman rank: P=0.046, r=0.283) and even stronger correlation under the linear assumption (Pearson's correlation: P=0.006, r=0.381), for both genders in a pooled analysis (Figure 2). Breakdown according to gender suggested that the pattern was retained or marginally retained in women (P=0.030, r=0.384 for linear and P=0.090, r=0.304 for non-linear), while in men the pattern was dissipated (P=0.108, r=0.392, P=0.203, r=0.315, respectively).

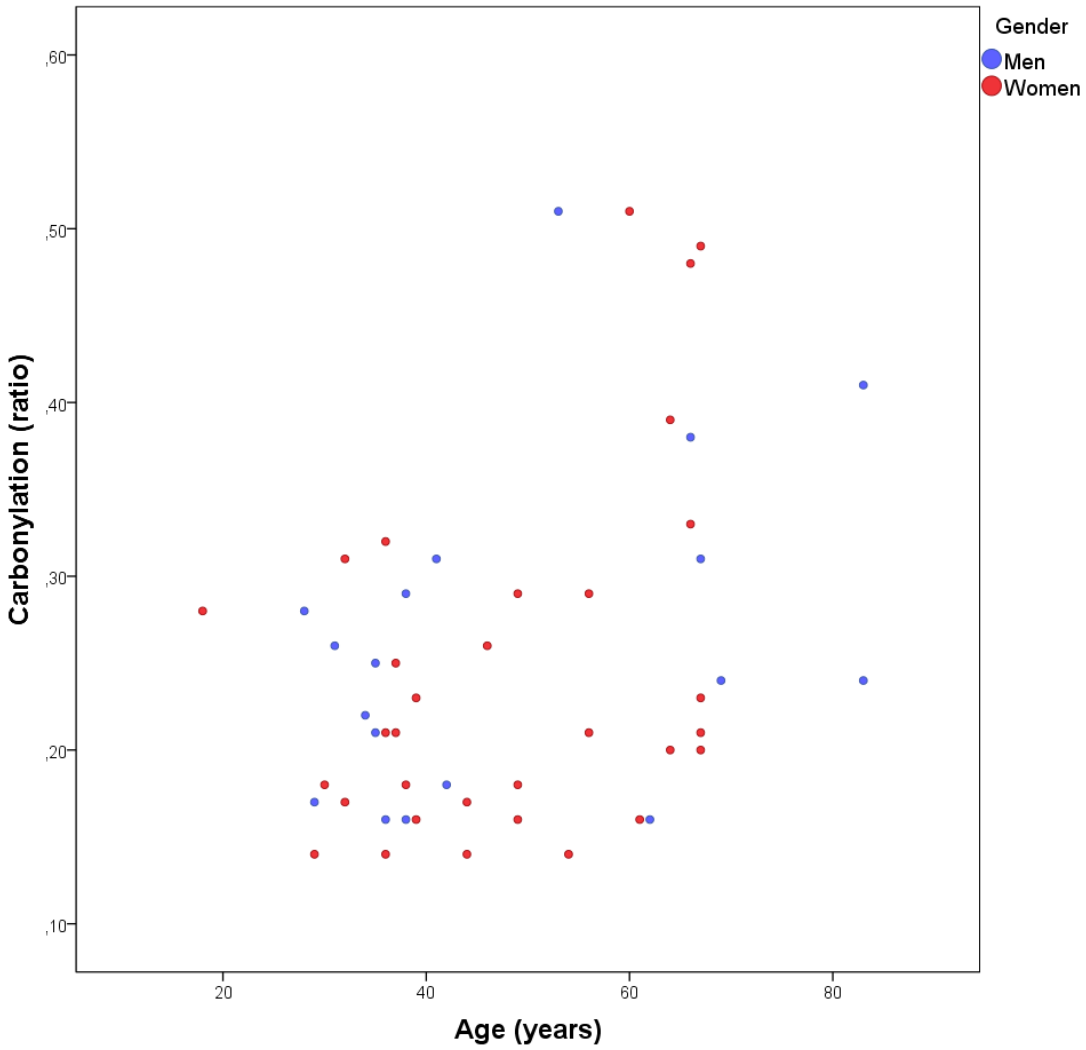

Figure 2. Scatterplot of age vs total 1D carbonylation, by gender

The more in-depth analysis had suggested the existence of two relatively separate sub-populations in men, broadly suggesting overall lower values in the younger men and more diverse values in elderly men (Figure 3). Fitting of a linear function suggested that the percent of variance explained was 15.3% (Figure 3). The similar pattern was observed in women, with possibly even stronger patter of greater carbonylation values in older women, with 14.7% of variance explained (Figure 4).

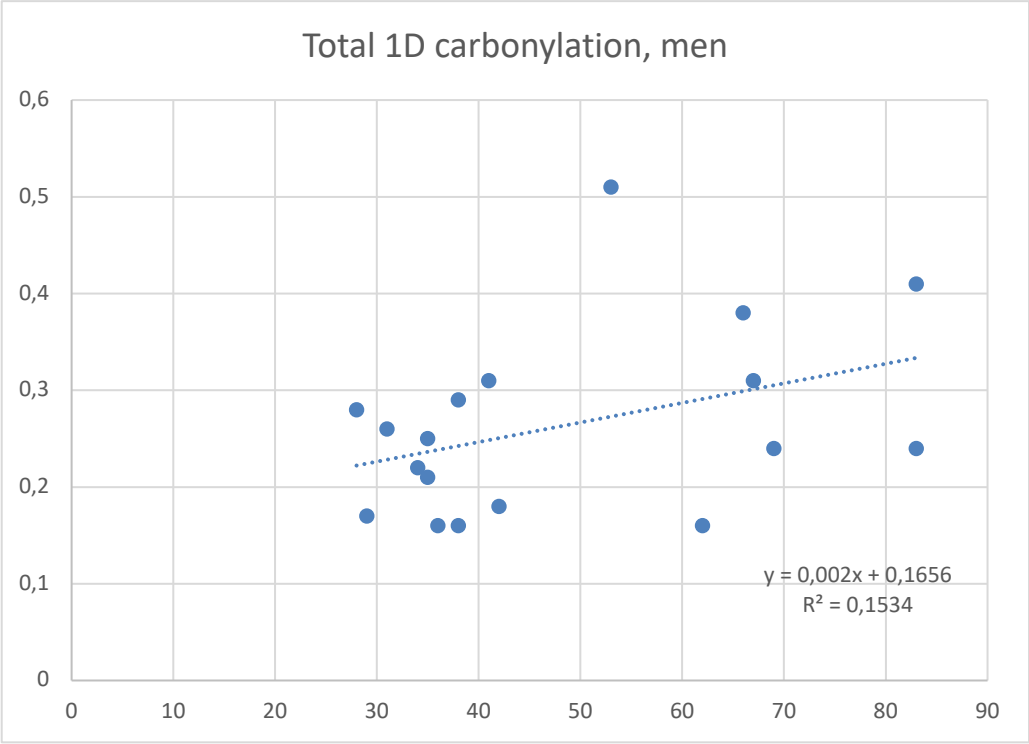

Figure 3. Scatterplot of total 1D carbonylation with age, men

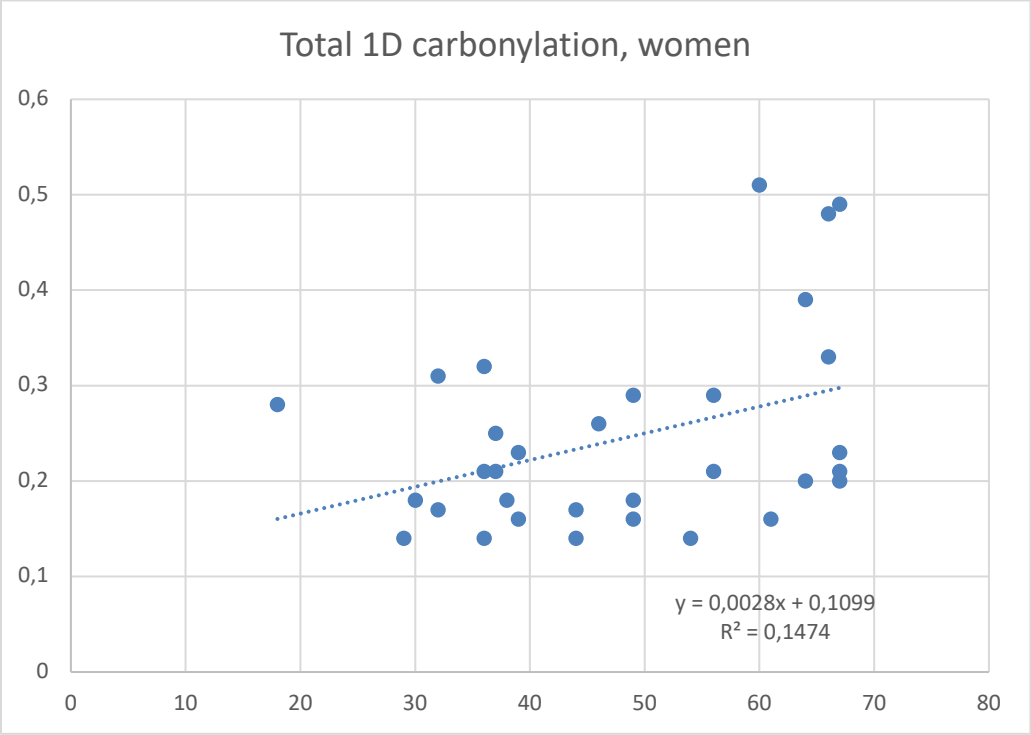

Figure 4. Scatterplot of total 1D carbonylation with age, women

Out of the large number of variables that were then correlated to carbonylation, several variables were significant, namely central augmentation index, pulse wave velocity and standard progressive matrices, besides brachial circumference and two more cognitive measures, verbal fluency test and Rey Auditory Verbal Learning Test, which were marginally significant (Table 3). Notably, directions of the three significant correlations were all in line with the expectations – higher carbonylation was correlated to worse result of standard progressive matrices, higher pulse wave velocity and central augmentation index (Table 3; all analyzed variables are presented in Table 4).

Table 3. Results of correlations of carbonylation with various variables

Variable	P	r
Cognition: standard progressive matrices	0.021	-0.345
Pulse wave velocity	0.031	0.305
Central augmentation indeks	0.042	0.289
Brachial circumference	0.050	0.279
Cognition: Rey Auditory Verbal Learning Test (RAVLT)	0.054	-0.289
Cognition: verbal fluency test (S)	0.097	-0.251

Table 4. Correlation coefficients of carbonylation vs. all other numeric variables

Variable	P	r
Height (cm)	0.691	-0.059
Weight (kg)	0.779	0.042
Finger-sternal length (cm)	0.155	-0.204
Waist circumference (cm)	0.186	0.190
Hip circumference (cm)	0.396	0.124
Brachial circumference (cm)	0.050	0.279
Head circumference (cm)	0.175	0.195
Neck circumference (cm)	0.428	0.115
Brachial width (cm)	0.705	0.080
Biceps skinfold, M1	0.171	-0.197
Biceps skinfold, M2	0.311	-0.146
Biceps skinfold, M3	0.497	-0.098
Triceps skinfold, M1	0.210	-0.180
Triceps skinfold, M2	0.353	-0.134
Triceps skinfold, M3	0.368	-0.130
Abdominal skinfold, M1	0.991	0.002
Abdominal skinfold, M2	0.955	0.008
Systolic BP, M1	0.878	-0.022
Systolic BP, M2	0.401	0.122
Diastolic BP, M1	0.467	-0.105
Diastolic BP, M2	0.117	0.224
Cognition: standard progressive matrices	0.021	-0.345
Pulse wave velocity	0.031	0.305
Cognition: SPM	0.021	-0.345
Cognition: verbal fluency (F)	0.185	0.201
Cognition: verbal fluency (A)	0.204	-0.193
Cognition: verbal fluency (S)	0.097	-0.251
Variable	P	r
Cognition: RAVLT	0.054	-0.289
Cognition: Mill Hill scale	0.504	-0.102
EPQ_P	0.885	-0.022
EPQ_E	0.807	-0.037
EPQ_N	0.354	-0.142
EPQ_L	0.137	0.225
Serum creatinine	0.714	0.053
Serum uric acid	0.216	0.178
Serum glucose	0.147	0.208
Serum cholesterol	0.597	0.077
Serum triglycerides	0.525	0.092
Serum HDL	0.689	-0.058
Serum LDL	0.567	0.083
Serum VLDL	0.472	0.104
Serum calcium	0.154	0.205
Serum albumin	0.616	-0.073
HbA1c	0.228	0.173
HbA1c_ifcc	0.236	0.171
Fibrinogen	0.662	-0.064
Order of birth	0.125	0.220
Number of siblings	0.525	0.096
Marital age	0.359	-0.151
Spouse age at marriage	0.058	-0.307
Number of children	0.844	0.029

The multivariate model with gender and carbonylation in prediction of chronological age yielded only 14.6% of variance, while a random split in two subset yielded variances of 5.3% and 7.8%, suggesting strongly underpowered analysis (Table 5). Notably, carbonylation was a significant predictor of age in the full model, yielding a P value of 0.007 (Table 5).

Table 5. Multivariate modelling with two steps of cross-validation, predicting age with gender and carbonylation

	Cross-validation 1		Cross-validation 2		All data	
	F	P	F	P	F	P
Model	5.373	0.030	8.044	0.010	11.082	0.002
Gender	0.001	0.997	0.578	0.455	0.031	0.860
Carbonylation	1.833	0.190	0.436	0.516	8.007	0.007
R2	7.8		5.3		14.6	

The simulation results suggested that the overall prediction was of moderate to large effect, with the existence of an exponential trend and the overall 59.06% of explained variance in comparison of the observed vs. predicted age (Figure 5). Simulation results indicated that the extent of difference of observed vs. predicted age was greater in elderly and in younger subjects, with significant quadratic trend (F=8.03, P=0.002; Figure 6).

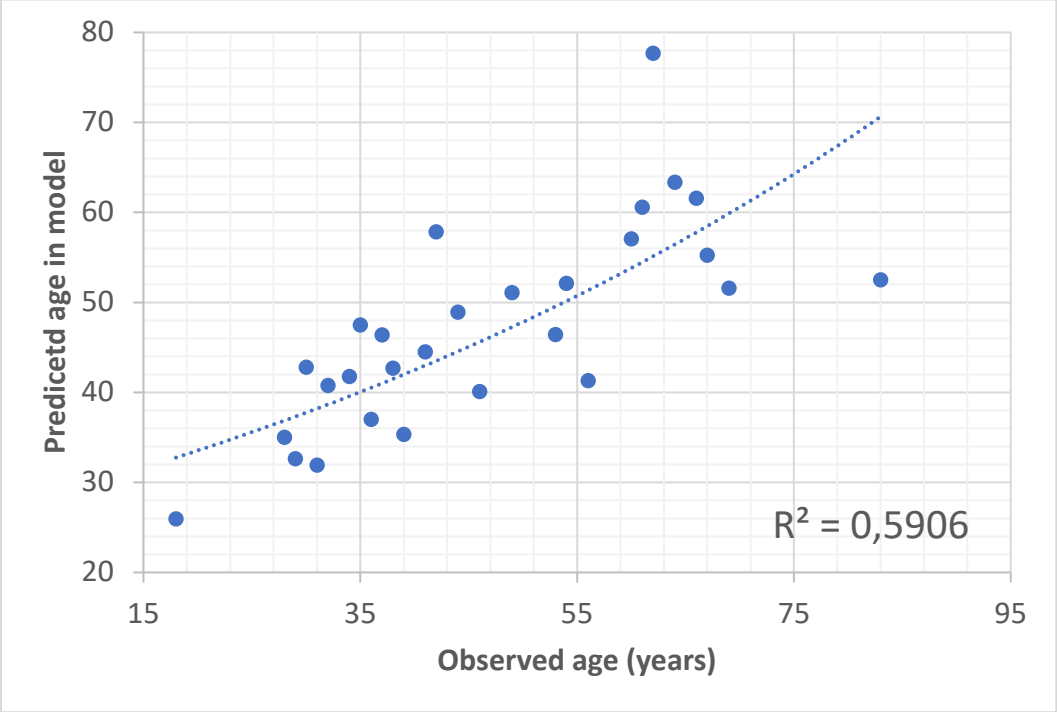

Figure 5. Comparison of the observed vs. predicted age from the simulation model

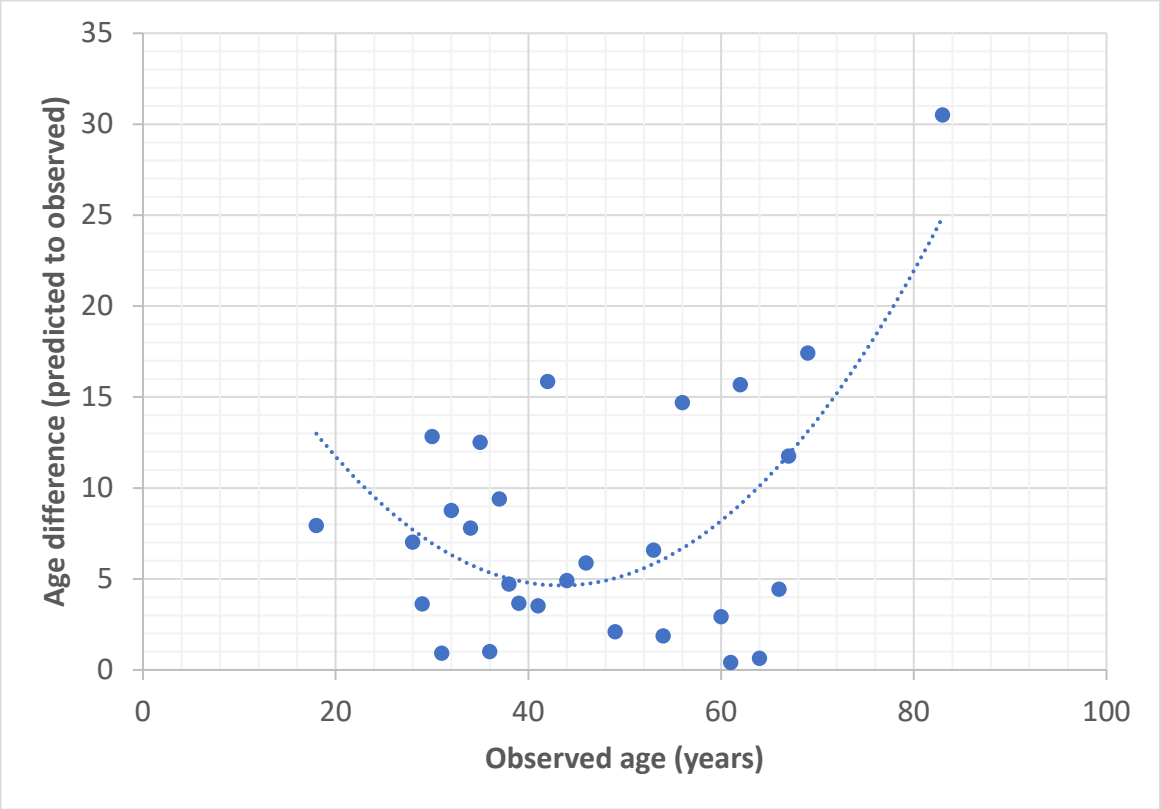

Figure 6. Scatterplot of age difference (predicted vs. observed) vs. observed age, with quadratic trend fit

5. DISCUSSION

The results of this study suggest that carbonylation is linked to chronological age, but that one-dimensional carbonylation has limited power in age prediction. This is based on the finding of certain amount of changes, but also on the substantial variation that was seen in the analyzed dataset. The extent of variation of carbonylation was rather large, while the percent of variance of predicted age that was explained by carbonylation was at best-case scenario close to only 15%. The results also show that the dataset was too small for more elaborate findings, suggesting that much larger datasets will be needed to refine these results and establish the possibility of use of carbonylation as a biomarker of ageing. These estimates were more reliable in the middle-aged, while they seemed to be less reliable in younger and more elderly. While this could simply be an artefact of the sample size, it could also point towards greater extent of biological variability in younger and in very old subjects.

The study shows that carbonylation values are more diverse in elderly men than the values in younger men. A reasonable explanation for that fact is that biological controlling mechanisms, namely antioxidants, which act like scavenger systems for ROS attenuate with age. This finding is consistent with a study from 2006 conducted by Rizvi *et al.*, in which they could point out that plasma antioxidant potential, which is the potential to neutralize oxidative stress, decreases as a function of age. They demonstrated it based on measurements displaying a decline of ferric reducing ability of plasma (FRAP) values (31).

Our results present a similar manner of carbonylation values in women, displaying lower values in younger women, but an even stronger erratic pattern of carbonylation in elderly women. This finding is possibly a consequence of the decline of estrogen levels in women after menopause to a level equal to those of age-matched men. C. Borrás et al. published 2003 a paper using an animal model with rats, demonstrating that mitochondria in females show a larger antioxidant gene expression and a smaller oxidative damage compared to males. This is a consequence of the activity of estrogens, which by means of intracellular signalling pathways upregulate nuclear expression of antioxidant enzymes, and thereby protecting the cells from the damaging effect of oxidative stress (32). In addition to that finding, C. Borrás et al. were able to show that besides upregulating antioxidant enzymes, estradiol targets estrogen receptors directly within the mitochondria, and as a consequence has a direct antioxidant and protective effect on isolated mitochondria (33). The previously mentioned studies explain our measurements that as soon the levels of estrogen fall

down after the start of menopause in women, the protective effect of estrogen ceases and the erratic pattern of carbonylation becomes stronger.

Notably, several interesting correlations were observed, with pulse wave velocity and cognition. The aortic pulse wave velocity (PWV) is the speed at which the arterial pulse travels through the circulatory system and is connected to the stiffness of the arteries. In his Croonian lecture from 1808, Thomas Young was the first scientist to describe the relationship between the elastic properties of arteries and the pulse wave velocity. Generally, the PWV is described by the Moens-Korteweg equation. In the process of aging, there are several changes occurring in the circulatory system, the central elastic arteries stiffen, diastolic pressure decline, and central systolic and pulse pressures are augmented because of elevated PWV and early return of reflected waves to the heart from the periphery (34).

The augmentation index (AI) is another tool, independent from the pulse wave velocity, which indirectly measures arterial stiffness. It has been scientifically shown that the AI increases with age (35). Furthermore research has shown that the compliance of arteries in the human circulatory system decreases with age (36) and that even in the absence of cardiovascular disease, the decline in arterial elasticity starts in childhood (37). To calculate the augmentation index, the augmentation pressure has to be divided by the pulse pressure x 100 to obtain a percentage. The augmentation pressure describes the contribution of the wave reflection to the systolic arterial pressure. It is acquired by the measurement of the reflected wave, which propagates from the periphery to the center. While aging, the AI increases with enhanced stiffening of the arteries, which leads to a faster propagation of the advancing pulse wave and a more quickly reflected wave (35).

Our results indicate a significant correlation between high levels of carbonylation and pulse wave velocity and carbonylation and the augmentation index. A higher and therefore more unfavorable level of PWV and AI correlate with higher carbonylation. Increased values for PWV and AI point to a decreased arterial compliance, which in turn is concomitant with increased age.

Standard Progressive Matrices (SPM) are valuable tools in the diagnostic of intelligence and evaluation of the intellect through abstract reasoning tasks. They nonverbally assess the cognitive capability of humans in a broad spectrum and are mostly independent of education, nationality and physical constitution of the test subject. Of three instruments, which are known as

the Raven's Progressive Matrices, SPM is the first and most widely used. The other two matrices are the Coloured Progressive Matrices (CPM) and the Advanced Progressive Matrices (APM).

We found out, that an increased level of carbonylation is associated with a worse performance in the Raven Standard Progressive Matrices test. Researchers from the university of Maastricht found out that also old age is associated to lower SPM test performance (38).

The most salient limitation of this study is small sample size, leading to underpowered analysis. Increasing the sample size would therefore be considered as the initial step in elucidating the link between carbonylation and ageing. Furthermore, we have measured only a single-dimensional carbonylation amount, which is the total sum of carbonylation of all serum proteins. It could easily be that the there are changes of specific proteins, suggesting that two-dimensional carbonylation is needed in terms of improvement of laboratory procedures. The cohort of subjects could also be standardized in a more elaborate way, in order to control for the most obvious confounding factors, such as diet, lifestyle and exercise.

6. CONCLUSION

1. Carbonylation correlates to chronological age, suggesting that it might be a useful biomarker of aging

2. However, as it is difficult to provide a real estimate of biological aging, the usefulness of carbonylation remains of limited use. We need to develop in two directions, namely measuring carbonylation in unhealthy subjects (aiming to see the association with morbidity), but also developing a protein-specific carbonylation estimate, which would allow a refined analysis.

3. Carbonylation was correlated to a number of variables that are known to deteriorate in elderly, like the pulse wave and cognition, further supporting the possibility to use it as a biomarker.

7. SUMMARY

Title: Plasma Protein Carbonylation as Biomarker for Estimate of Age

Objectives: The main aim of this study was to investigate the role of carbonylation in ageing and its role as biomarker for estimation of age

Materials and methods: The data from the 10.001 Dalmatians biobank were used, focusing on the 50 healthy subjects from the island of Korčula. One-dimensional carbonylation of plasma was measured, using HPLC. The results were correlated to a number of available variables from this dataset.

Results: The results of this study suggest that carbonylation is linked to chronological age, but that it has limited power in individual age prediction (Spearman rank: P=0.046, r=0.283, Pearson's correlation: P=0.006, r=0.381). Furthermore, carbonylation values were more diverse in elderly than the young men, the percent of variance explained was 15.3%. Similar to men, carbonylation values are lower in younger women, but show a stronger erratic pattern of carbonylation in elderly women, explained with a variance of 14.7%. Additionally, there is correlation between high levels of carbonylation and Pulse Wave Velocity and the Augmentation Index. A higher and therefore more unfavorable level of PWV and AI correlate with higher carbonylation and older age (PWV: P=0.031, r=0.305, AI: P=0.042, r=0.289). Last but not least, an increased level of carbonylation is associated with a worse performance in the Raven Standard Progressive Matrices test (P=0.054, r=-0.289).

Conclusion: Carbonylation correlates chronological age, but either larger sample sizes or the use of two-dimensional carbonylation is required before individual age prediction can be more reliable.

8. CROATIAN SUMMARY

Naslov: Karbonilacija proteina plazme kao biomarkera za procjenu dobi

Ciljevi: Glavni cilj ove studije bio je istražiti ulogu karbonilizacije u starenju i mogućnosti korištenja za procjenu starosti

Materijali i metode: Upotrijebljeni su podaci iz biobanke 10.001 Dalmatinac, s naglaskom na 50 zdravih ispitanika s otoka Korčule. Jednodimenzionalna karbonilacija plazme mjerena je pomoću HPLC-a. Rezultati su korelirani s raznim mjerenjima iz ovog projekta.

Rezultati: Rezultati ovog istraživanja sugeriraju da je karbonilacija povezana sa starenjem, ali da ima ograničenu snagu u predviđanju starosti pojedinca, (Spearman rank: P=0.046, r=0.283, Pearson's correlation: P=0.006, r=0.381). Nadalje, vrijednosti karbonilacije bile su raznovrsnije kod starijih muškaraca, postotak objašnjenje varijacije iznosio je 15,3%. Slično muškarcima, vrijednosti karbonilacije niže su kod mlađih žena, ali u starijim dobnim skupinama pokazuju mnogo veću raznolikost, objašnjeno varijacijom od 14,7%. Osim toga, postoji korelacija između visokih nivoa karbonilacije i brzine pulsnog vala (PWV: P=0.031, r=0.305, AI: P=0.042, r=0.289). Posljednje, ali ne i najmanje važno, povećana razina karbonilacije povezana je s lošijom rezultatom kognitivnih testova (P=0.054, r=-0.289).

Zaključak: Karbonilacija korelira sa starenjem, ali za provedbu osobne procjene dobi potrebna je ili veća veličina uzorka ili upotreba dvodimenzionalne karbonilizacije.

9. REFERENCES

1. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 1956;11(3):298-300.

2. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids. 2003;25(3-4):207-18.

3. Baker GT, 3rd, Sprott RL. Biomarkers of aging. Exp Gerontol. 1988;23(4-5):223-39.

4. Burkle A, Moreno-Villanueva M, Bernhard J, Blasco M, Zondag G, Hoeijmakers JH, et al. MARK-AGE biomarkers of ageing. Mech Ageing. 2015;151:2-12.

5. Oeppen J, Vaupel JW. Demography. Broken limits to life expectancy. Science. 2002;296(5570):1029-31.

6. Brown GC. Living too long: the current focus of medical research on increasing the quantity, rather than the quality, of life is damaging our health and harming the economy. EMBO Rep. 2015;16(2):137-41.

7. Sebastiani P, Thyagarajan B, Sun F, Schupf N, Newman AB, Montano M, et al. Biomarker signatures of aging. Aging Cell. 2017;16(2):329-38.

8. Anstey KJ, Lord SR, Smith GA. Measuring human functional age: a review of empirical findings. Exp Aging Res. 1996;22(3):245-66.

9. Mitnitski AB, Graham JE, Mogilner AJ, Rockwood K. Frailty, fitness and late-life mortality in relation to chronological and biological age. BMC Geriatr. 2002;27;2:1.

10. Karasik D, Demissie S, Cupples LA, Kiel DP. Disentangling the genetic determinants of human aging: biological age as an alternative to the use of survival measures. J Gerontol A Biol Sci Med Sci. 2005;60(5):574-87.

11. Suzuki YJ, Carini M, Butterfield DA. Protein carbonylation. Antioxid Redox Signal. 2010;12(3):323-5.

12. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med. 2006;10(2):389-406.

13. Levine RL, Moskovitz J, Stadtman ER. Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. IUBMB Life. 2000;50(4-5):301-7.

14. Dalle-Donne I, Giustarini D, Colombo R, Milzani A, Rossi R. S-glutathionylation in human platelets by a thiol-disulfide exchange-independent mechanism. Free Radic Biol Med. 2005;38(11):1501-10.

15. Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, Lungarella G, et al. Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. Mass Spectrom Rev. 2005;24(1):55-99.

16. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta. 2003;329(1-2):23-38.

17. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. Clin Chem. 2006;52(4):601-23.

18. Vistoli G, De Maddis D, Cipak A, Zarkovic N, Carini M, Aldini G. Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. Free Radic Res. 2013;47 Suppl 1:3-27.

19. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552(Pt 2):335-44.

20. Cheeseman KH, Slater TF. An introduction to free radical biochemistry. Br Med Bull. 1993;49(3):481-93.

21. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010;4(8):118-26.

22. Gianazza E, Crawford J, Miller I. Detecting oxidative post-translational modifications in proteins. Amino Acids. 2007;33(1):51-6.

23. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev. 2014;94(3):909-50.

24. Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. Arch Biochem Biophys. 2002;397(2):342-4.

25. Bartz RR, Piantadosi CA. Clinical review: oxygen as a signaling molecule. Crit Care. 2010;14(5):234.

26. Lushchak VI. Free radicals, reactive oxygen species, oxidative stress and its classification. Chem Biol Interact. 2014;224:164-75.

27. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 2nd ed. Oxford: Clarendon Press; 1989.

28. Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol. 1997;82(2):291-5.

29. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol. 2014;24(10):R453-62.

30. Rudan I, Marusic A, Jankovic S, Rotim K, Boban M, Lauc G, et al. "10001 Dalmatians:" Croatia launches its national biobank. Croat Med J. 2009;50(1):4-6.

31. Rizvi SI, Jha R, Maurya PK. Erythrocyte plasma membrane redox system in human aging. Rejuvenation Res. 2006;9(4):470-4.

32. Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. Free Radic Biol Med. 2003;34(5):546-52.

33. Borras C, Gambini J, Lopez-Grueso R, Pallardo FV, Vina J. Direct antioxidant and protective effect of estradiol on isolated mitochondria. Biochim Biophys Acta. 2010;1802(1):205-11.

34. Mitchell GF, Parise H, Benjamin EJ, Larson MG, Keyes MJ, Vita JA, et al. Changes in arterial stiffness and wave reflection with advancing age in healthy men and women: the Framingham Heart Study. Hypertension. 2004;43(6):1239-45.

35. Fantin F, Mattocks A, Bulpitt CJ, Banya W, Rajkumar C. Is augmentation index a good measure of vascular stiffness in the elderly? Age Ageing. 2007;36(1):43-8.

36. Bulpitt CJ, Cameron JD, Rajkumar C, Armstrong S, Connor M, Joshi J, et al. The effect of age on vascular compliance in man: which are the appropriate measures? J Hum Hypertens. 1999;13(11):753-8.

37. Laogun AA, Gosling RG. In vivo arterial compliance in man. Clin Phys Physiol Meas. 1982;3(3):201-12.

38. Van der Elst W, Ouwehand C, van Rijn P, Lee N, Van Boxtel M, Jolles J. The shortened Raven Standard Progressive Matrices: item response theory-based psychometric analyses and normative data. Assessment. 2013;20(1):48-59.

10. CURRICULUM VITAE

Personal Data:

Name and Surname: Laura Langert

Date and place of birth: 28.01.1993 in Bocholt, Germany

Citizenship: German

Address: Walter-Hallstein-Weg 4, 46399 Bocholt

E-mail: laura.langert@web.de

Education:

2012-2018 University of Split School of Medicine, Split, Croatia

2009-2012 Berufskolleg am Wasserturm, Bocholt

2006-2009 Israhel-van-Meckenem Realschule, Bocholt

Languages:

German, mother tongue

English, fluent

French, B2 level